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# New Approaches to Immunotoxicity Testing

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**New approaches to immunotoxicity testing are reviewed and discussed. A method of activating T-cells *in vivo* is presented which circumvents artifacts due to viability effects encountered with *in vitro* mitogen assays. The use of adoptive transfer approaches to combine the advantages of *in vitro* manipulation with *in vivo* function assays is discussed relative to natural killer cells. The need for an *in vitro* metabolic activation step coupled to other *in vitro* immunologic assays is discussed.**

## Introduction

Previous papers have demonstrated the importance of the immune system with regard to the well-being of all animals. It has also been clearly demonstrated that chemicals can modify the immune response, some for the benefit of the animal, e.g., immunopharmaceuticals, and some in potentially harmful ways, e.g., chemical wastes and environmental pollutants. Adequate coverage of the new approaches to immunotoxicity testing would require a separate symposium. The volume of new methods being applied to all sorts of immunologic investigations is enormous, and the list of literature reports increases monthly. Thousands of compounds and their effects on the immune system require examination, and rapid, economical screening procedures would certainly benefit industry as well as health and regulatory agencies.

## General Approaches to Testing for Immunotoxicity

Table 1 shows the basic ways in which immune response tests may be carried out. In the first category of tests the cells involved in a particular immune response are exposed directly to the chemical *in vitro* and the test for immune function is also carried out *in vitro*. Although this approach is the most economical in terms of time and animals, it has some problems, most notably: metabolic alteration of the compound may not be accounted for; distribution of the chemical into the immune compartment is assumed; and cells are more susceptible to damage in the relatively dilute *in vitro* microenvironment. A compromise approach in terms of realism and economy calls for *in vivo* exposure to the chemical followed by *in vitro* testing of immune function of various lymphoid tissue. This is clearly the most common approach currently used in immunotoxicity studies. The major advantages of this approach are that distribution of the chemical to the immune compartment is realistic and metabolism of the compound may occur naturally. This approach will be discussed further. Lastly, animals may be exposed to the chemical *in vivo* and immune functions tested *in vivo*. Although this is the most

**Table 1. General immune response test scheme.**

| Toxicant exposure | Test environment |
|---|---|
| *In vitro* | *In vitro* |
| *In vivo* | *In vitro* |
| *In vivo* | *In vivo* |

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realistic approach, it too has definite drawbacks: large numbers of animals are usually required, owing to the variability of immune responses even in syngeneic populations of mice; and at present many of the *in vitro* manipulations and tests cannot be duplicated *in vivo*.

Returning to the *in vivo* exposure/*in vitro* analysis scheme, some disadvantages with this approach have recently been noted.

## Lymphoproliferative Assays

Figure 1*a* depicts the standard lymphoproliferative assay (or mitogen assay) as currently performed. Although chemical contact occurs *in vivo*, it was thought that preparation of cells for *in vitro* culture might remove the chemical; thus chemical and activator (or mitogen) might not be present simultaneously. Conversely, cells might be metabolically or physically damaged by the chemical, making them appear nonfunctional in the more hostile *in vitro* situation, but compensatory mechanisms *in vivo* might permit normal functions to occur. To test these hypotheses, we used staphylococcal enterotoxin A (SEA), a T-cell specific mitogen (*1*) with low toxicity to the mouse and with the added advantage of a broad plateau of activation rather than the sharp dose-related peaks commonly seen with the lectin mitogens concanavalin A or phytohaemmagglutinin (*2*). SEA was shown to be an *in vivo* T-cell activator (*3*). The only *in vitro* culture required was a 4-hr period for $^{3}$H-thymidine uptake (Fig. 1*b*). Thus, chemical and activator could be present simultaneously *in vivo*. During these studies, we noticed that certain agents administered *in vivo*, such as antithymocyte serum (ATS), which disrupts T-cell membrane integrity, showed a greater suppressive effect on *in vitro* mitogen responsiveness at far lower doses than were required to suppress *in vivo* activation of T cells by SEA.

Table 2 shows the effects of ATS on *in vitro* survival of cells, as determined by vital dye staining. Spleen cells from ATS-treated animals were 97-98% viable at the time of harvest. Four hours of culture had only a slight negative effect on *in vitro* survival of ATS-treated cells. The rate of cell death of 48-hr cultured cells (the routinely used LP assay time) was greatly accelerated by ATS pretreatment compared with the *in vitro* death rate of controls. Magnification of the apparent damage in the *in vitro* culture is advantageous because greater sensitivity is attained; however, further work has shown that realism may be compromised. Experiments have indicated that the shorter the *in vitro*

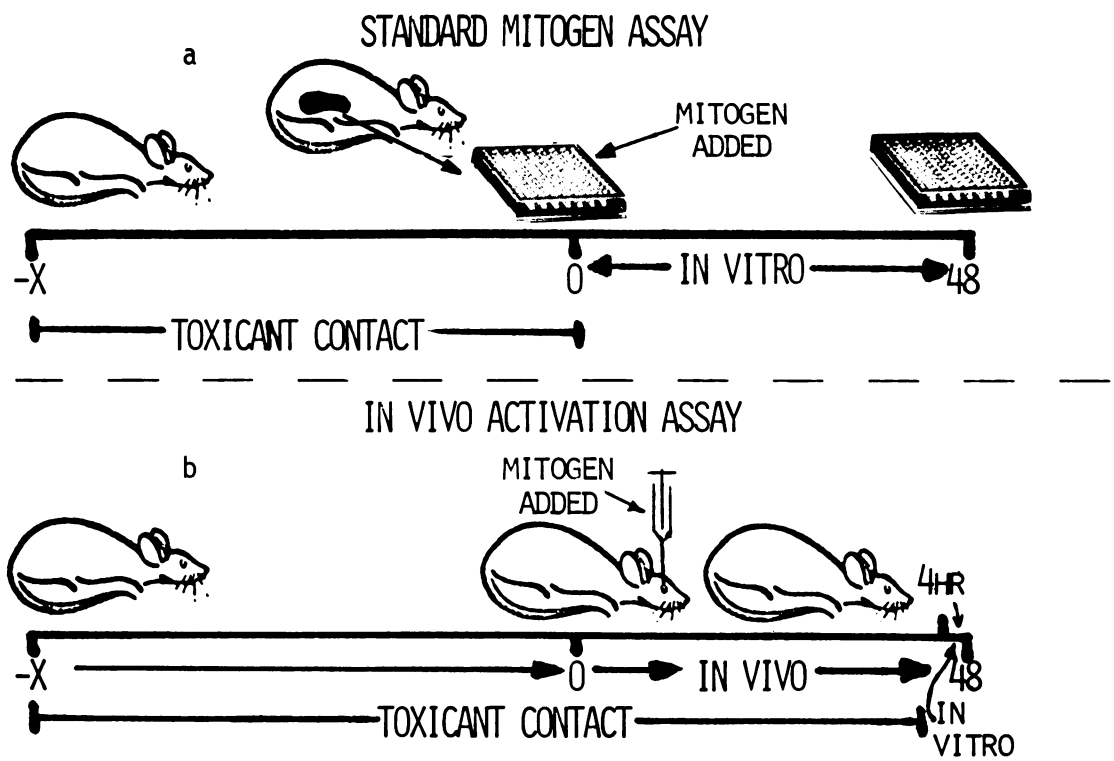


FIGURE 1. (*a*) Standard lymphoproliferative assay. (*b*) *In vivo* activation assay (*3*).

**Table 2. Viability of ATS-treated and untreated mouse spleen cells after 4 and 48 hr *in vitro*.**

| Incubation time *in vitro*, hr | Viability by trypan blue dye exclusion, % (mean ± SD)[a] | |
|---|---|---|
| | Control for ATS | ATS-treated[b] |
| 4 | 97 ± 1.1 | 95.2 ± 1.5[c] |
| 48 | 84 ± 5.1 | 50.7 ± 7.9[c] |

[a] $n = 12$ for each point

[b] Treatments carried out as described in text.

[c] $p < 0.01$ by Student's $t$-test performed on arc-sines of proportion of viability.

incubation time, the less the chance of amplified effects on viability.

## *In Vitro* Manipulation-Adoptive Transfer

In a recent report concerning natural killer cells, Kasai et al. (4) demonstrate how the concept of short *in vitro* culture time might be applied. Natural killer (NK) cells have become the focus of many recent studies on antitumor activities of the immune system because they appear to be one of the first-line defenses against virus- or chemical-transformed cells and have a functional counterpart in humans. Kasai et al. attempted to demonstrate that cell subpopulations which expressed NK activity *in vitro* were actually responsible for NK activity *in vivo*. The antitumor role of NK cells *in vivo* had not yet been conclusively shown. The experimental approach taken by these investigators demonstrates how several new technologies could be applied to immunotoxicologic investigations. Figure 2 depicts the separation scheme used by Kasai et al. Their procedure, which could be applied to chemical-exposed animals as well, was as follows.

Spleen cells were removed from mice. Some cells were placed in plastic dishes coated with antimouse Fab antibody; in this way, immunoglobulin (Ig)-bearing cells (B-cells) were enriched and non-Ig-bearing cells were removed. These populations were retained for testing. Other spleen cells were passed through a nylon wool column known to retain Ig-bearing B-cells. Thus, T-cells bearing Thy 1 antigen were enriched as were other non-Ig-bearing cells. Some of these cells were treated with anti-Thy 1.2 antibody + C′, to specifically lyse the mature, Thy

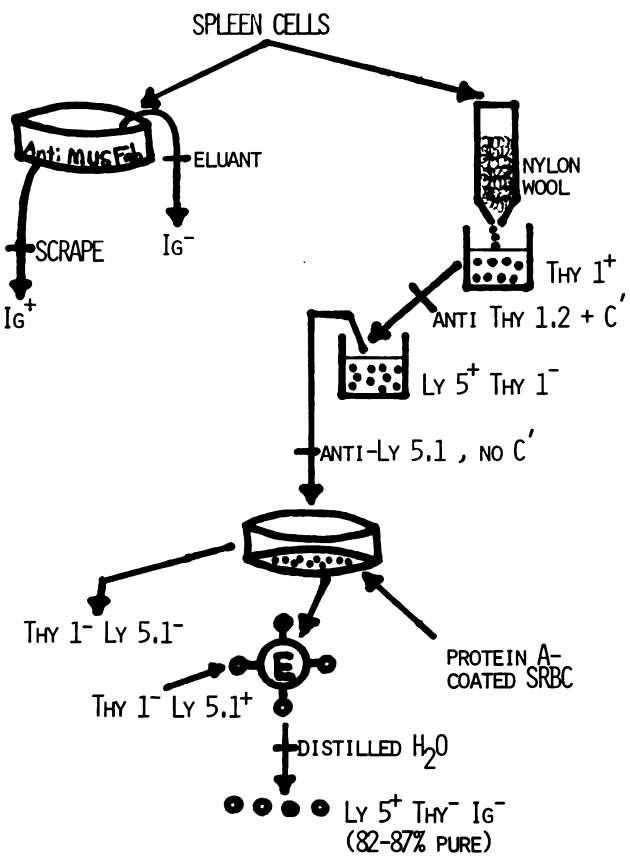


FIGURE 2. Separation of spleen cell populations used by Kasai et al. (4).

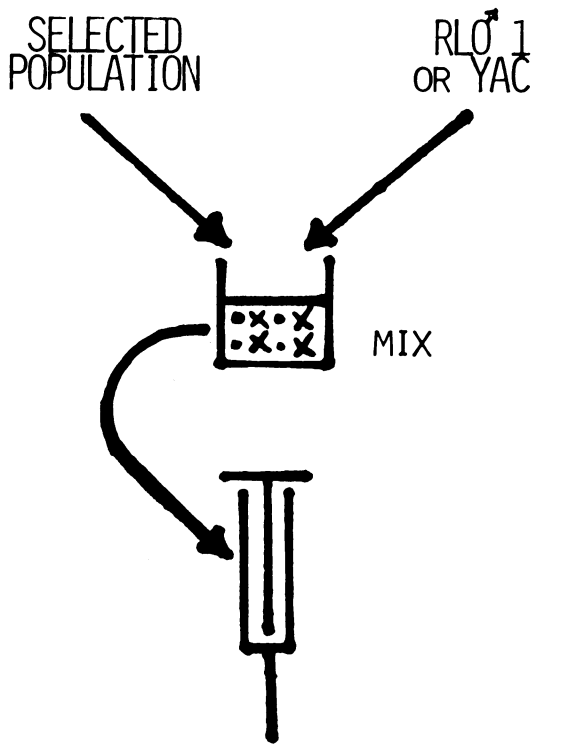


FIGURE 3. Adoptive transfer of tumor cells and separated spleen cell subpopulations used by Kasai et al. (4).

Table 3. Ability of different lymphocyte sets to prevent lymphoma growth *in vivo*.

| Spleen cell population | Cell number ($\times 10^6$) | Tumor incidence[a] | |
|---|---|---|---|
| | | Host = BALB/c, tumor = RLol | Host = A/J, tumor = YAC |
| A None | -- | 22/22 | 16/16 |
| B Unselected | 10 | 8/8 | 8/8 |
| C Nylon-passed cells | 5 | 14/15 | 15/15 |
| D $Thy1^+$ cells | 5 | 8/8 | 8/8 |
| E $Ig^+$ cells | 5 | 16/16 | 16/16 |
| F $Thy1^-$ cells | 5 | 19/22 | 22/22 |
| G $Thy1^-Ig^-Ly5^+$ cells | 5 | 1/12 | 0/12 |
| H Group G treated with anti-Ly5.1 + C | 5 | 7/8 | 7/8 |

[a]Results of three individual experiments (*4*).

1-bearing T cells. Monoclonal antibody technology currently assures the purity of such antibody preparations. The Thy $1^-$ cells were found to have Ly 5 surface antigen by fluorescent antibody methods. The Thy $1^-$ Ly $5^+$ cells were coated with anti-Ly 5 antibody, but without complement because no lysis was desired. The coated, viable cells were placed in dishes with protein A-coated sheep erythrocytes. Protein A specifically binds to the Fc portion of antibody molecules; the Ly $5^+$ cells were bound to erythrocytes by the protein A-antibody bridge and could easily be separated from nonbound cells. The erythrocytes were lysed by distilled $H_2O$ shock and the Ly $5^+$ cells were freed and retained for testing. These cells were 82-87% pure as determined by fluorescence testing with specific antisera. The total *in vitro* time for these cell separations was relatively short, probably less than 4 hr.

Figure 3 shows how the isolated cell populations from each step of separation were mixed with RL1 or YAC tumor cells and adoptively transferred to appropriate mice. The *in vitro* manipulated cells were thus returned to an *in vivo* environment.

Table 3 depicts the results of the adoptive transfer conditions. Spleen cells alone, although they contained the NK cells, would not demonstrate protection, probably because of insufficient numbers of NK cells. Not until the Ly $5^+$ Thy $1^-$ cells were highly enriched could protection be seen, but then it was quite remarkable. Further, lysis of this population by Ly 5 antisera showed that the population carrying this surface antigen was definitely involved in protection from the tumor. Such manipulations are impossible in a totally *in vivo* situation.

By such separations, NK cells or other desired populations could be isolated *in vitro*, exposed to chemical treatment to assess direct effects, particularly if *in vitro* metabolic activation could be carried out, and returned to the natural *in vivo* environment in a syngeneic host for testing. Likewise, such tests could be done on cells from chemical-pretreated animals.

Combining *in vitro* manipulation with adoptive transfer and *in vivo* functional analysis is one way in which immunotoxicologic studies might be carried out using currently available methodology and averting possible erroneous conclusions caused by problems associated with *in vitro* survivability.

## New Approaches to Screening for Immunotoxicants

Recently, Kutz et al. (*5*) have applied the Mishell-Dutton *in vitro* antibody-forming cell system to screening a large number of environmentally relevant compounds. Many of these chemicals were shown to be immunosuppressive but directly cytotoxic to lymphoid cells *in vitro* at suppressive levels (Table 4). These chemicals included the organotins, several heavy metals and polychlorinated biphenyls. Others, shown in Table 5, were suppressive but not cytotoxic. Most notably, azathioprene was suppressive *in vitro* at levels easily attained *in vivo*

Table 4. Effects of various chemicals on the direct PFC response and viability of Mishell-Dutton cultures.

| Compound | 90% PFCSD, μg/culture (mean ± SD)[a] | 50% VRD, μg/culture (mean ± SD)[a] |
|---|---|---|
| Cytotoxic | | |
| DBTC | 0.092 ± 0.003 | 0.06 ± 0.01 |
| DPTC | 0.46 ± 0.006 | 0.27 ± 0.06 |
| DOTC | 3.80 ± 0.15 | 1.18 ± 0.05 |
| DETC | 4.77 ± 0.16 | 3.72 ± 0.06 |
| DMTC | 5.21 ± 0.35 | 3.53 ± 0.03 |
| Propyl gallate | 8.95 ± 0.13 | 5.08 ± 0.36 |
| Mercuric chloride | 8.77 ± 0.35 | 8.11 ± 1.14 |
| Cadmium chloride | 8.90 ± 0.24 | 9.43 ± 0.42 |
| Aroclor 1254 | 17.25 ± 1.60 | 17.92 ± 0.40 |
| Aroclor 1248 | 19.02 ± 0.33 | 17.72 ± 0.19 |
| Nickel chloride | 45.42 ± 0.09 | 37.93 ± 0.80 |

[a]90% PFCSD = 90% plaque-forming cell suppressive dose compared to nontreated positive controls; 50% VRD = 50% viability reducing dose. Data are expressed as means ± standard deviations of triplicate cultures (*5*).

**Table 5. Effects of various chemicals on the direct PFC response and viability of Mishell-Dutton cultures.**

| Compound | 90% PFCSD, μg/culture (mean ± SD)[a] | 50% VRD, μg/culture (mean ± SD)[a] |
|---|---|---|
| Immunosuppressive (at noncytotoxic doses) | | |
| Azathioprine (Imuran) | 0.51 ± 0.19 | 16.39 ± 0.52 |
| Gallic acid | 7.56 ± 0.07 | < 300 |
| Methyl paraben | 98.90 ± 0.95 | 265.36 ± 11.08 |
| Dextran sulfate | 101.17 ± 5.85 | < 300 |
| Vanillin | 199.75 ± 6.64 | < 300 |
| No detectable effect | | |
| Chromic chloride | < 300 | < 300 |
| Cyclophosphamide | < 300 | < 300 |
| Lead chloride | < 300 | < 300 |
| Sodium chloride (182 μg Cl) | < 300 | < 300 |

and with similar suppression kinetics. Thus, some degree of predictability was achieved with this system. Cyclophosphamide also had no effect on this system, owing to the lack of the required microsomal activation of this chemical. The need for an *in vitro* activation step in this potentially useful procedure is discussed by Tucker (*6*). Vos (*7*) and Boorman (*8*) also report on new methods for assessing the effects of chemicals on the adult immune system and the developing immune system, respectively.

## Conclusion

Immunology as related to toxicology is an exciting field of research. We are currently learning which methods are applicable to toxicity testing and improving and adapting others to make them suitable. While no one magic test will suffice, it may be possible in the very near future to report the development of more rapid, reliable, economical screening methods for a wide range of immune functions which have predictive value to the whole animal.

### REFERENCES


1. Smith, B. G., and Johnson, H. M. The effect of staphylococcal enterotoxins on the primary *in vitro* immune response. J. Immunol. 115: 575-578 (1975).
2. Archer, D. L., Smith, B. G., Ulrich, J. T., and Johnson, H. M. Immune interferon induction by T-cell mitogens involves different T-cell subpopulations. Cell. Immunol. 48: 420-426 (1979).
3. Archer, D. L., Smith, B. G., and Peeler, J. T. Activation of murine T-lymphocyte DNA synthesis *in vivo* by staphylococcal enterotoxin A: Applicability to immunotoxicity testing. IRCS Med. Sci. 8: 457 (1980).
4. Kasai, M., Leclerc, J. C., McVay-Boudreau, L., Shen, F. W., and Cantor, H. Direct evidence that natural killer cells in nonimmune spleen cell populations prevent tumor growth in vivo. J. Exptl. Med. 149: 1260-1264 (1979).
5. Kutz, S. A., Hinsdill, R. D., and Weltman, D. J. Evaluation of chemicals for immunomodulatory effects using an in vitro antibody-producing assay. Environ. Res. 22: 368-378 (1980).
6. Tucker, A. N., Sanders, V. M., Hallett, P., Kauffmann, B., and Munson, A. E. *In vitro* immunotoxicological assays for detection of compounds requiring metabolic activation. Environ. Health Perspect. 43: 123-127 (1982).
7. Vos, J. G., Krajnc, E. I., and Beekhof, P. Use of the enzyme-linked immunosorbent assay (ELISA) in immunotoxicity testing. Environ. Health Perspect. 43: 115-121 (1982).
8. Boorman, G. A., Luster, M. I., Dean, J. H., and Campbell, M. L. Assessment of myelotoxicity caused by environmental chemicals. Environ. Health Perspect. 43: 129-135 (1982).